Article

The effect of a gelatin ß-tricalcium phosphate sponge loaded with mesenchymal stem cells (MSC), bone morphogenic protein-2, and platelet-rich plasma (PRP) on equine articular cartilage defect

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Abstract — We evaluated the curative efficacy of a gelatin ß-tricalcium phosphate (ß-TCP) sponge loaded with mesenchymal stem cells (MSC), bone morphogenic protein-2 (BMP-2), and platelet-rich plasma (PRP) by insertion into an experimentally induced osteochondral defect. A hole of 10 mm diameter and depth was drilled in the bilateral medial femoral condyles of 7 thoroughbred horses, and into each either a loaded sponge (treatment) or a saline-infused ß-TCP sponge (control) was inserted. After 16 weeks, defects were examined by computed tomography, macroscopic analyses, and histological analyses. The median subchondral bone density and macroscopic subscores for joint healing were significantly higher in the treatment legs ($P < 0.05$). Although there was no significant difference in total histological scores between groups, hyaline cartilaginous tissue was observed across a wider area in the treatment group. Equine joint healing can be enhanced by inserting a BMP-2-, MSC-, and PRP-impregnated ß-TCP sponge at the lesion site.

Résumé — L'effet d'une éponge de phosphate ß-tricalcique de gélatine imbibée de cellules souches mésenchymateuses (CSM), d'une protéine-2 morphogénétique osseuse et d'un plasma riche en plaquettes (PRP) sur un défaut de cartilage articulaire équin. Nous avons évalué l'efficacité curative d'une éponge de phosphate ß-tricalcique de gélatine (ß-TCP) imbibée de cellules souches mésenchymateuses (CSM), d'une protéine-2 morphogénétique osseuse (P2MO) et d'un plasma riche en plaquettes (PRP) en l'insérant dans un défaut ostéo-cartilagineux induit par expérimentation. Un trou de 10 mm de diamètre et de profondeur a été percé dans les condyles fémoraux médiaux bilatéraux de 7 pur-sang et, chez chaque cheval, une éponge imbibée (traitement) ou une éponge ß-TCP infusée d'une solution saline (témoin) a été insérée. Après 16 semaines, les défauts ont été examinés par tomographie par ordinateur, analyses macroscopiques et analyses histologiques. La densité osseuse sous-chondrale et les sous-notes médianes de la guérison des articulations étaient significativement supérieures dans les jambes traitées ($P < 0{,}05$). Même s'il n'y avait pas de différences significatives au niveau des notes histologiques totales entre les groupes, le tissu cartilagineux hyalin a été observé sur une région plus vaste dans le groupe de traitement. La guérison des articulations équines peut être améliorée en insérant une éponge ß-TCP imbibée de P2MO, de CSM et de PRP sur le site de la lésion.

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Introduction

Many equine joint diseases, such as osteochondritis dissecans and subchondral bone cysts, are accompanied by cartilage damage and osteochondral defects. Cartilage is an avascular tissue composed of cells with low mitotic potential surrounded by an extensive extracellular matrix, tissue properties that significantly restrict healing capacity (1,2). Moreover, regenerated cartilage is not hyaline cartilage but more fragile fibrocartilage (3,4). Therefore, the promotion of hyaline cartilage regeneration is a desirable event.

Tissue engineering techniques that incorporate viable mitotic cells, growth factors, and growth-promoting substrates into biocompatible scaffolds may accelerate osteochondral defect

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healing and hyaline cartilage regeneration (5–7). Different combinations of cells and growth factors can be supplied to the defect site by inserting a 3-dimensional (3D) scaffold that promotes growth factor release, cell adhesion, proliferation, and differentiation (8,9).

Multipotent mesenchymal stem cells (MSC) can differentiate into both osteocytes and chondrocytes to promote osteochondral regeneration (3,4,10). Bone morphogenetic protein-2 (BMP-2) and transforming growth factor-ß1 (TGFß1) are also known to facilitate osteochondral regeneration (11,12). Platelets contain many growth factors such as platelet-derived growth factor (PDGF) and TGFß1 in α-granules. Thus, platelet-rich plasma (PRP) may further improve cartilage regeneration by augmenting the supply of growth factors for chondrocyte precursor proliferation and differentiation (12–15). However, growth factors have very short biological half-lives, necessitating a specialized drug delivery system for localized and sustained application (16).

Gelatin hydrogel is a biocompatible material that has proven effective for drug delivery and as a cell growth substrate. Gelatin hydrogel and growth factors form stable electrostatic interactions, and growth factors are released as the hydrogel is degraded by hydrolysis (16). Gelatin hydrogel can be formed into microspheres, sheets, and porous sponges to suit specific clinical applications (16). Porous gelatin hydrogel sponges can act as 3D scaffolds for cell proliferation and differentiation. However, the porous structure can also reduce mechanical strength. In fact, the compression modulus of gelatin hydrogel sponge is insufficient for cell scaffold applications (17). In order to overcome this disadvantage, a stronger gelatin ß-tricalcium phosphate (ß-TCP) sponge was developed that shows no change in porous structure (18).

Repair of osteochondral defects requires the regeneration of 2 biologically and functionally distinct tissues, cartilage, and subchondral bone. Scaffolds impregnated with stem cell and multiple growth factors may accelerate the healing of both tissues, but the scaffold properties must allow the controlled release of chemically distinct growth factors and provide an environment conducive to cell proliferation and differentiation, conditions difficult to meet using a uniform sponge material. Thus, a multilayered structure may be necessary for simultaneous cartilage and subchondral bone regeneration to repair osteochondral defects (1,7,19). We speculated that a multilayered gelatin ß-TCP sponge scaffold incorporating MSCs, BMP-2, and PRP-derived growth factors could promote both subchondral bone growth and hyaline cartilage regeneration. The aim of this study was to evaluate the therapeutic efficacy of such a multilayered scaffold for the repair of an experimentally created osteochondral defect in the equine medial femoral condyle.

Materials and methods

Horses

Seven healthy thoroughbred horses [1 male, 6 female, mean weight ± standard deviation (SD): 388.9 ± 123.5 kg, mean age ± SD: 3.3 ± 3.7 years] were used in this study. All horses were stall-rested throughout the study period. They were fed 3500 g of dried grass twice daily for the first 2 wk after surgery and then fed an ordinary diet (900 g of oats, 300 g of bran, and 3500 g of dried grass twice daily) for the remainder of the study period. This study was approved by the Experimental Animal Committee of Obihiro University of Agriculture and Veterinary Medicine.

Preparation of gelatin hydrogel microspheres and sponges

Gelatin hydrogel microspheres (GMs) [isoelectric point (IEP): 5.0, water content: 97.8%] were prepared from gelatin hydrogel with an IEP of 5.0 (Nitta Gelatin Co. Osaka, Japan) according to the method of Sasaki et al (20). Gelatin ß-TCP sponges [IEP: 9.0, water content: 97.8%, ß-TCP content: 50%, porosity: 95.9%, pore size: 179.1 ± 27.8 μm] were prepared from gelatin hydrogel with an IEP of 9.0 (Nitta Gelatin Co.) and ß-TCP (Taihei Chemical, Osaka, Japan) according to the method of Takahashi et al (17). The gelatin ß-TCP sponge was formed into a columnar shape (diameter: 10 mm, length: 10 mm) to conform to the bone defect and then sterilized by ethylene oxide.

Preparation of mesenchymal stem cell with osteogenic or chondrogenic differentiation capacity

Bone marrow was aspirated from the sternum as previously described (21) and cultured at 0.1 mL per culture dish (Nunclon™Δ 90 mm; Thermo Fisher Scientific, Franklin, Massachusetts, USA) in 10.2 mL Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Tokyo, Japan) supplemented with 1.8 mL fetal bovine serum (BioWest, Nuaille, France) and 0.12 mL penicillin–streptomycin solution (10 000 units penicillin and 10 mg streptomycin/mL; Sigma-Aldrich). The culture medium was changed 3 d after plating and then every second day. During each medium change, non-adherent cells were washed out with PBS and discarded to select proliferating MSC. Cells were maintained in culture for 2 wk before they were removed from the plate and infused into the sponge. In brief, 5 h before preparation of the osteochondral defect, MSCs were dissociated from the culture dishes by 2-min incubation in 5 mL of 0.25% trypsin-EDTA saline (Mediatech, Manassas, Virginia, USA) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were harvested and centrifuged (4°C, 250 × *g*, 10 min). The supernatant was discarded and 500 μL of culture medium were added to the MSC pellet. This MSC suspension was stored at 4°C until it was used. Two other MSC suspensions were prepared, 1 with osteogenic differentiation medium and the other with chondrogenic differentiation medium to confer osteogenic and chondrogenic capacity. Osteogenic capacity was confirmed by Von-kossa staining and real time polymerase chain reaction (RT-PCR) (22). Chondrogenic capacity was confirmed by immunostaining for type 2 collagen (Collagen Staining Kit 2.0; Chondrex, Redmond, Washington, USA) and by RT-PCR (23,24).

Preparation of platelet-rich plasma and bone morphogenetic protein-2 solution

Twenty-four hours before preparation of the osteochondral defect, blood was collected and PRP was prepared following the method described by Nagae et al (25). Venous blood was

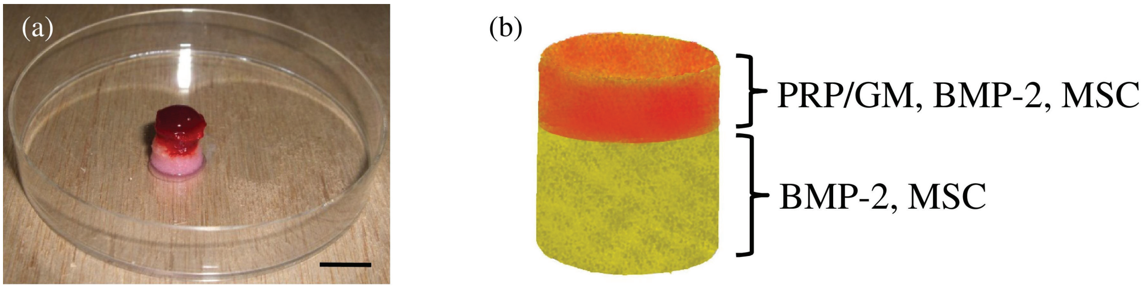

Figure 1. The ß-TCP sponge impregnated with bone morphogenetic protein-2 (BMP-2), mesenchymal stem cells (MSCs), and platelet-rich plasma incorporated onto gel microspheres (PRP/GM). The appearance (a) and structure (b) of the impregnated sponge are shown. The impregnated sponge was designed as a bilayer by maintaining the PRP/GM within the upper layer so that growth factors in PRP were released only from the upper layer, whereas BMP-2 was released from the entire sponge. Scale bar = 10 mm.

collected from the jugular vein and mixed with the anticoagulant citrate dextrose. This blood mixture was centrifuged (10°C, 250 × *g*, 10 min) and the plasma and buffy coat layers were collected and centrifuged again (10°C, 1000 × *g*, 15 min). The upper layer of plasma was discarded, and the lower plasma layer (1 mL) and the buffy coat were collected and used as PRP. Platelet enrichment relative to whole blood was confirmed by an automatic blood cell counter (Celltac α MEK-6358; Nihon Kohden, Tokyo, Japan). After PRP preparation and platelet counting, 600 μL were added to 10 mg of GM (PRP/GM) and the PRP/GM suspension was mixed well by pipetting. A solution of BMP-2 (0.01 μg/μL) (Recombinant human BMP-2; Peprotech, Rocky Hill, New Jersey, USA) was prepared concurrently following an established method (26). In brief, 10 μg of BMP-2 was dissolved in 500 μL saline. This solution was transferred to a filter tube and centrifuged (12 000 × *g*, 4°C) to concentrate the solution to 50 μL. The BMP-2 solution was divided into two 15 μL-aliquots (3 μg of BMP-2 per aliquot) and diluted to a total volume of 300 μL with saline.

Impregnation of gelatin hydrogel sponges

An aliquot of the BMP-2 solution (300 μL containing 3 μg of BMP-2) was impregnated into each gelatin ß-TCP sponge and these BMP-2/sponges were stored at 4°C until they were used. One hour before the surgery, the MSC suspensions and the PRP/GM mixture were impregnated into the BMP-2/sponge. The MSC suspension was seeded by static submersion (22), followed by dripping of the PRP/GM mixture slowly onto the sponge (1 drop/5 s) using an 18-gauge needle to ensure that the viscous PRP/GM was restricted to the upper layer of the BMP-2/sponge (impregnated sponge, Figure 1). Localization of PRP/GM to the top layer was confirmed prior to insertion of the sponge into the osteochondral defect site. Other gelatin ß-TCP sponges of the same dimensions were impregnated with saline for control experiments.

Preparation of the osteochondral defect

Each horse was premedicated intravenously (IV) with medetomidine hydrochloride (Domitor; Nippon Zenyaku Kogyo, Fukushima, Japan), 4 μg/kg body weight (BW). Five minutes later, diazepam (Horizon; Astellas, Tokyo, Japan) 0.03 mg/kg BW, IV, was administered. Guaifenesin (ALPS Pharmaceutical, Gifu, Japan), 25 mg/kg, IV, was then rapidly infused until the horse became ataxic. After injection of guaifenesin, the anesthetic thiamylal sodium (Isozole; Nichiiko, Toyama, Japan), 4 mg/kg BW was administered IV. The trachea was then intubated and the horse was placed on a surgical table. Anesthesia was maintained by the inhalation of isoflurane (Isoflu; Dainippon Sumitomo Pharmaceutical, Osaka, Japan) in oxygen.

The horse was placed in dorsal recumbency and the limb was flexed with the stifle at 90°. An arthroscope was inserted into the femorotibial joint following the established method (27). The joint surface was observed to confirm that there were no preexisting cartilage lesions. A drill (NACHi 10.0 mm; Fujikoshi, Toyama, Japan) was inserted between the middle patellar and medial patellar ligaments using a drill guide and a hole (diameter: 10 mm, depth: 10 mm) was drilled in the medial femoral condyle. After removal of the bone fragments by rinsing and suction of the joint cavity, an impregnated sponge or control sponge was pushed into the drilled hole (PRP/GM layer at the articular surface in the treatment group) using a flat-tipped metal bar. Sponge placement and fixation were confirmed by arthroscopy. The joint capsule and skin were sutured separately. The contralateral leg was treated in the same manner except that the other sponge type (impregnated or saline-treated) was inserted. Legs were randomly selected to be the treatment or control side for each horse.

Postoperative treatment

Postoperative medication consisted of the antibiotic cephalothin sodium (Coaxin; Tobishi Pharmaceutical, Tokyo, Japan), 20 mg/kg BW, IV, q12h for 5 d, and 2 non-steroidal anti-inflammatory drugs: flunixin meglumine (Banamine; Dainippon Sumitomo Pharma), 1.1 mg/kg BW, IV, q12h for 3 d, and phenylbutazone (Bute Tabs; Vedco, St. Joseph, Missouri, USA), 2 mg/kg BW, PO, q24h for 4 d. Postoperative evaluations included general clinical measurements (body temperature, heart rate, respiration rate, heat around the surgical site, joint effusion, gastrointestinal motility, capillary refill time), blood tests (complete blood cell counts, serum electrolyte levels), and pulse quality at the palmar digital artery. These examinations were conducted every day for the first 2 wk and once a week thereafter. Mobility was examined during a weekly walk and trot "in hand" over a hard surface. All examinations for lameness were conducted by the same veterinarian for the entire 16-week postoperative study period.

Table 1. Macroscopic evaluation of regenerated cartilage (1 = worst, 4 = excellent)

Score	Surface texture	Void filled	Tissue integration
1	Rough and red (< 50%)	< 50%	< 50%
2	50% to 75%	50% to 75%	50% to 75%
3	75% to 100%	75% to 100%	75% to 100%
4	Smooth and white (= 100%)	Completely filled (100%)	Completely integrated (100%)

Computed tomographic evaluation

All horses were euthanized 16 wk after the surgery. Each horse was given an intravenous injection of 5 μg/kg BW medetomidine hydrochloride (premedication), 10 mg/kg BW thiamylal sodium, and 50 mg/kg BW guaifenesin (anesthetic overdose) followed by exsanguination by cutting the common carotid artery. During exsanguination, unconsciousness was assessed by the loss of the corneal reflex. Death was determined by cardiac arrest.

The computed tomography (CT) system (Asteion™ Super 4; Toshiba Medical Systems Corporation, Tochigi-ken, Japan) was calibrated using an air and water phantom prior to imaging of defect sites in treatment and control legs. Both hind legs were dissected from the body by cutting the femur 20 cm from the head and each stifle was examined using CT. Three-dimensional image processing software (Virtual Place Advance; Aze, Tokyo, Japan) was used for postprocessing of CT data within a 10 mm deep × 9.8 mm wide rectangular region of interest centered at the subchondral bone defect. The CT attenuation value [in Hounsfield units (HU)] was calculated and compared to that measured from the (untreated) lateral femoral condyle.

Histological analysis

After CT examination, repair of the femorotibial joint (cartilage regeneration) was first examined by gross inspection followed by histochemical staining of tissue sections. The joints were first examined to detect macroscopic abnormalities such as adhesions and osteophyte formation. Macroscopic changes were quantified by 3 indices: surface texture, tissue growth in the lesion (void filling), and tissue integration (Table 1), each graded from 1 (worst) to 4 (excellent) according to the classification scheme of Wilke et al (3). Osteochondral tissue samples together with the associated cartilage were then retrieved from the area surrounding the defect. In brief, tissue cubes (20 mm × 20 mm × 20 mm) were cut from around the defect using a surgical saw, fixed in 15% formalin, decalcified in 99% formic acid, embedded in paraffin wax, and sectioned sagittally across the osteochondral defect. Sections were then stained with hematoxylin and eosin and safranin-O/fast green and subjected to immunostaining for type 2 collagen. The histological condition of the cartilage was graded according to Wilke et al (Table 2) (3). The condition of the subchondral bone at the defect site was graded 0–5 according to the percentage of compact bone, where: 0 = < 20% compact bone, 1 = 20% to 40%, 2 = > 40% to 60%, 3 = > 60% to 80%, 4 = > 80% to 99%, and 5 = 100% compact bone (24). All histological evaluations were conducted by the same veterinarian, who was blind to the treatment (use of the impregnated or control sponge).

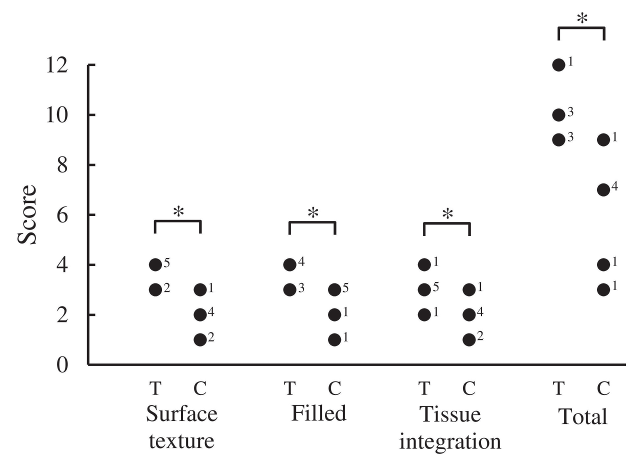

Figure 2. Total macroscopic scores and subscores (surface texture, void filled, tissue integration) for joint defects treated with the impregnated sponge (treatment, T) and the saline-infused sponge (control, C). Macroscopic scores were significantly higher in the treatment group. The numbers beside data points denote the number of specimens with that particular score. $^{*}P < 0.05$ (Wilcoxon signed-rank test)

Statistical analysis

All data were analyzed using the Statcel2 statistical software package (OMS Publishing, Saitama, Japan). No data set was normally distributed; therefore, all are expressed as median and range. Differences in medians between groups were evaluated for statistical significance using the Wilcoxon signed-rank test. A P of < 0.05 was considered statistically significant.

Results

The median platelet enrichment in PRP used to treat sponges was 3.0-fold (range: 2.2–6.6) compared with whole blood (enriched median platelet count of 42.0×10^4/μL, range: 20.5×10^4 to 97.5×10^4/μL). Impregnated sponges were treated with a median number of 10.6×10^6 MSC (range: 6.9×10^6 to 12.4×10^6). Mesenchymal stem cells used in this study were confirmed to have osteogenic or chondrogenic capacity.

In the first few days after surgery, mild heat around the surgical site and mild effusion at the medial femorotibial joint were observed in both treated stifles of all horses, but these signs of inflammation gradually decreased in severity and were virtually absent by 1 wk after the surgery. All horses exhibited a mild increase in peripheral white blood cell count (up to 12 000 cells/μL) after surgery, but this also improved within 5 d and normalized within 10 d. No other complication, such as lameness during a walk over a hard surface and abnormal blood test values, were observed during the 16-week postoperative study period.

The median CT attenuation value of the untreated lateral femoral condyle was 573.2 HU (481.4 to 749.0 HU) compared with 436.2 HU (238.3 to 637.9 HU) at the medial condyle defect sites implanted with impregnated sponges and 209.2 HU (130.5 to 389.7 HU) at the defect sites implanted with saline-infused sponges. The median CT attenuation value was significantly higher in the treatment group compared with

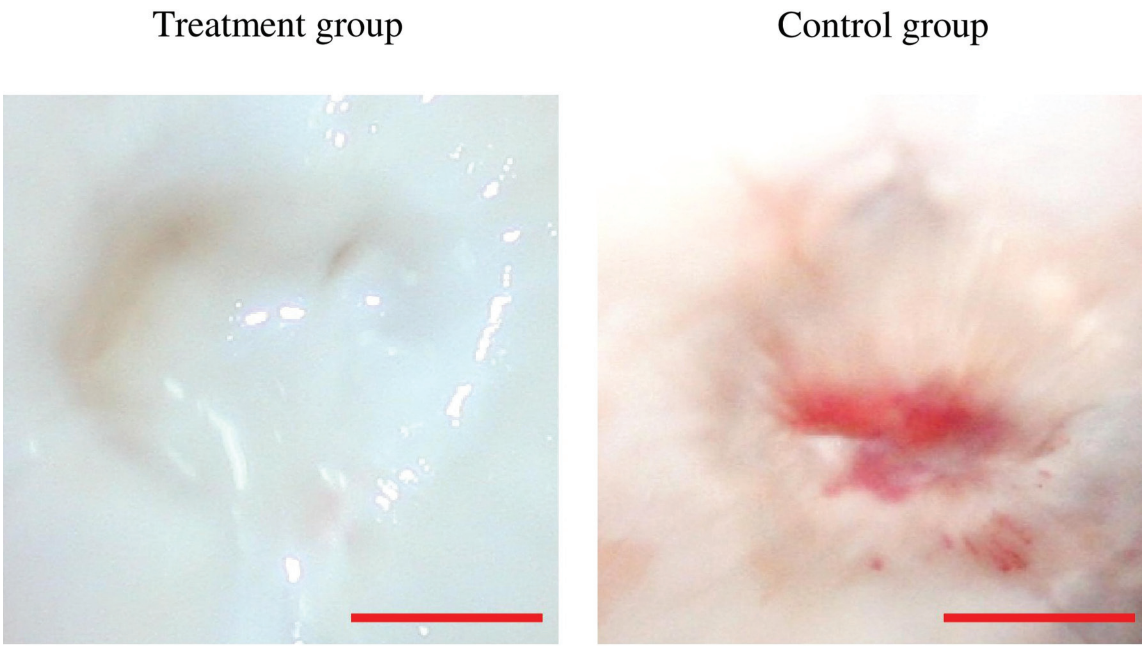

Figure 3. Macroscopic appearance of the treated and control joints of horse No. 5. On the treatment side (left), the subchondral bone in the drill hole was covered by white, smooth tissue (macroscopic subscores: surface texture = 4, filled = 3, tissue integration = 3, total = 10). On the control side (right), the drill hole was covered with red, rough tissue (macroscopic subscores: surface texture = 2, filled = 3, tissue integration = 2, total = 7). Scale bar = 5 mm.

the control group ($P < 0.05$), indicating accelerated deposition of radiodense bone and cartilage tissues at sites treated with impregnated sponges. Furthermore, the treatment side had a higher CT attenuation value than the control side for each horse.

At the macroscopic level, no abnormal findings such as adhesions and osteophyte formation in the femorotibial joint were observed. Both the total macroscopic score and macroscopic subscores (for surface texture, void filling, and tissue integration) were significantly higher in the treatment group ($P < 0.05$), indicating improved healing in horses treated with the impregnated sponges (Figure 2). In one horse (No. 1), the defect site in the treatment leg could not be distinguished from the surrounding tissue. Therefore, this horse was given an overall score of 4 for the treatment leg. Typical macroscopic images (from horse No. 5) are shown in Figure 3.

In contrast to these macroscopic indices of healing, there were no significant differences in the median total histological score and median subscores between groups (Figure 4). Again, we were unable to recover a sample from the treatment leg of horse No. 1 because the defect site could not be distinguished from the surrounding tissue. Typical stained sections used in these histological analyses are presented in Figure 5. Although the scores did not differ significantly, both safranin-O/fast green staining of cartilage and immunostaining for type 2 collagen covered a larger area in the treatment group than in the control group. In contrast to these indices of cartilage repair, the median histological score for subchondral bone density was significantly higher in the treatment group compared with the control group (treatment group: median score of 3, range 1–4; control group: median of 1, range 0–4; $P < 0.05$).

Discussion

Joint healing is often unsatisfactory due to the inherently low reparative potential of cartilage. However, healing can be improved by supplying stem cells, growth factors, and growth substrates at the site of injury. Previous reports have demonstrated that BMP-2 was effective in bone and cartilage regeneration because it promoted differentiation of MSC into osteocytes and chondrocytes and stimulated the synthesis of the chondrocyte extracellular matrix (11,12). Platelet-rich plasma contains both PDGF and TGFß1 and was also shown to be effective in cartilage regeneration because it promoted chondrocyte proliferation and proteoglycan and type-2 collagen synthesis (12). Other studies, however, found no beneficial effect of PRP on bone regeneration (28–31). Therefore, we speculated that BMP-2 and PRP at the site of cartilage regeneration and BMP-2 alone at the site of subchondral bone regeneration are favorable for osteochondral regeneration.

Gelatin ß-TCP sponge consisting of gelatin hydrogel with an IEP of 9.0 proved to be an effective scaffold material for BMP-2 because the electrostatic interaction required for drug delivery was suitable for BMP-2 (17). However, other growth factors, including PDGF and TGFß1, showed only weak electrostatic interactions with gelatin hydrogel of IEP 9.0 (16,32). Two previous reports suggested that a gelatin hydrogel with an IEP of 5.0 is required for effective delivery of PRP-derived growth factors (16,32). Thus, PRP and BMP-2 cannot be incorporated simultaneously within the same gelatin ß-TCP sponge. However, gelatin microspheres with an IEP of 5.0 were shown to be suitable delivery vehicles for PRP (33). Furthermore, microspheres increased the viscosity of PRP so that it remained localized at the site of application on the gelatin ß-TCP sponge. These

Table 2. Histologic scoring system (0 = worst, 4 = excellent)

Analysis	Score	Qualifications
Depth of defect filled	4	91% to 100%
	3	76% to 90%
	2	51% to 75%
	1	26% to 50%
	0	< 25%
Chondrocyte predominance	4	All
	3	Base and sides
	2	Sides only
	1	None
Chondrocyte organization	4	Normal base arrangement
	3	Multiple isogenous groups or columns in base
	2	Several groups in base
	1	None
Tidemark presence	4	Completely reformed
	3	Obvious but incomplete
	2	Barely perceptible
	1	None
Perilesional chondrocyte cloning	4	None
	3	1–3 adjacent to edge
	2	4–6 adjacent to edge
	1	> 6 at edges
Integration with adjacent perimeter cartilage	4	Fully integrated
	3	Cleft on one side
	2	Cleft on both sides
	1	Empty defect
Integration with subchondral bone	4	Full attachment
	3	Cleft across < 50% base
	2	Cleft across 50% to 90% base
	1	No attachment
Surface architecture (fibrillation)	4	Normal smooth surface
	3	Slight fibrillation
	2	Moderate fibrillation
	1	Severe fibrillation
Saflanin O staining relative to adjacent cartilage	4	> 75%
	3	25% to 75%
	2	5% to 25%
	1	None
Collagen type 2 predominance	4	< 75%
	3	25% to 75%
	2	5% to 25%
	1	None

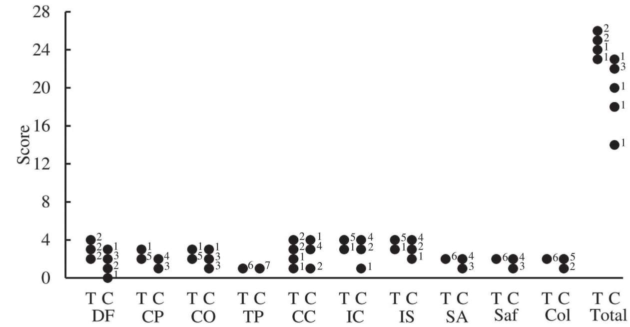

Figure 4. Total histological scores and subscores for the treated and control joints. There were no significant differences in histological scores between the treatment and control groups. Numbers beside each data point denote the number of specimens with that particular score. T – treatment group; C – control group; DF – depth of defect filled; CP – chondrocyte predominance; CO – chondrocyte organization; TP – tidemark presence; CC – perilesional chondrocyte cloning; IC – integration with adjacent perimeter cartilage; IS – integration with subchondral bone; SA – surface architecture (fibrillation); Saf – Safranin-O staining relative to adjacent cartilage; Col – collagen type 2 predominance.

properties allowed the fabrication of bilayered impregnated sponges that maintain PRP/GM within the upper layer. Using this particular drug delivery system, BMP-2 and PRP could be retained at the site of cartilage regeneration, with BMP-2 also released around the subchondral regeneration area (Figure 1). BMP-2 has been shown to promote bone regeneration in horses (34,35), and we previously demonstrated that BMP-2 is still effective when incorporated into gelatin ß-TCP sponges (26). Furthermore, combining BMP-2 with MSCs enhanced bone formation more effectively than did either treatment alone (18,36,37). Therefore, we speculated that gelatin ß-TCP sponges incorporating BMP-2, MSCs, and PRP in a bilayered structure would provide optimal bone and cartilage regeneration and thus improve joint healing.

The CT attenuation value of subchondral bone was significantly higher in the treatment group than in the control group, indicating higher bone density (20). Therefore, subchondral bone appeared to have regenerated faster in the presence of the BMP-2-, MSC-, and PRP-impregnated sponge. We assume that this improvement stemmed from both the targeted release of growth factors and the addition of MSCs, which would differentiate into subchondral bone under the action of BMP-2 and into cartilage under the influence of BMP-2 and PRP-derived growth factors.

Both the total macroscopic score and the macroscopic subscores for surface texture, void filling, and tissue integration were significantly higher in the treatment group, suggesting accelerated cartilage regeneration. Defects were filled, likely due to enhanced subchondral bone regeneration by the lower layer (BMP-2 and MSC layer) of the impregnated sponge. The smoothness of the cartilage surface was also significantly improved, likely due to enhanced cartilage regeneration by the upper layer (PRP/GM, BMP-2, and MSC layer) of the impregnated sponge. Rough joint surfaces decrease cartilage smoothness and increase the friction coefficient, leading to damage of the counter joint surface (38,39). Thus, application of these impregnated sponges may improve short-term osteochondral regeneration and long-term joint recovery.

In addition to these macroscopic indices, the density of the subchondral bone as measured histologically was also significantly higher in the treatment group, suggesting accelerated subchondral bone regeneration, consistent with the higher CT attenuation values measured in the treatment group. Ebihara et al (40) concluded that poor regeneration of subchondral bone was associated with weak safranin-O staining of the implanted chondrocytes and that better cartilage regeneration was accompanied by successful subchondral bone regeneration. Moreover, inhibition of subchondral bone resorption resulted in successful cartilage transplantation (41). Subchondral bone has an important role in stress absorption and in joint shape maintenance (42). Insufficient stress absorption increases the risk of cartilage damage during recovery, and poor repair of

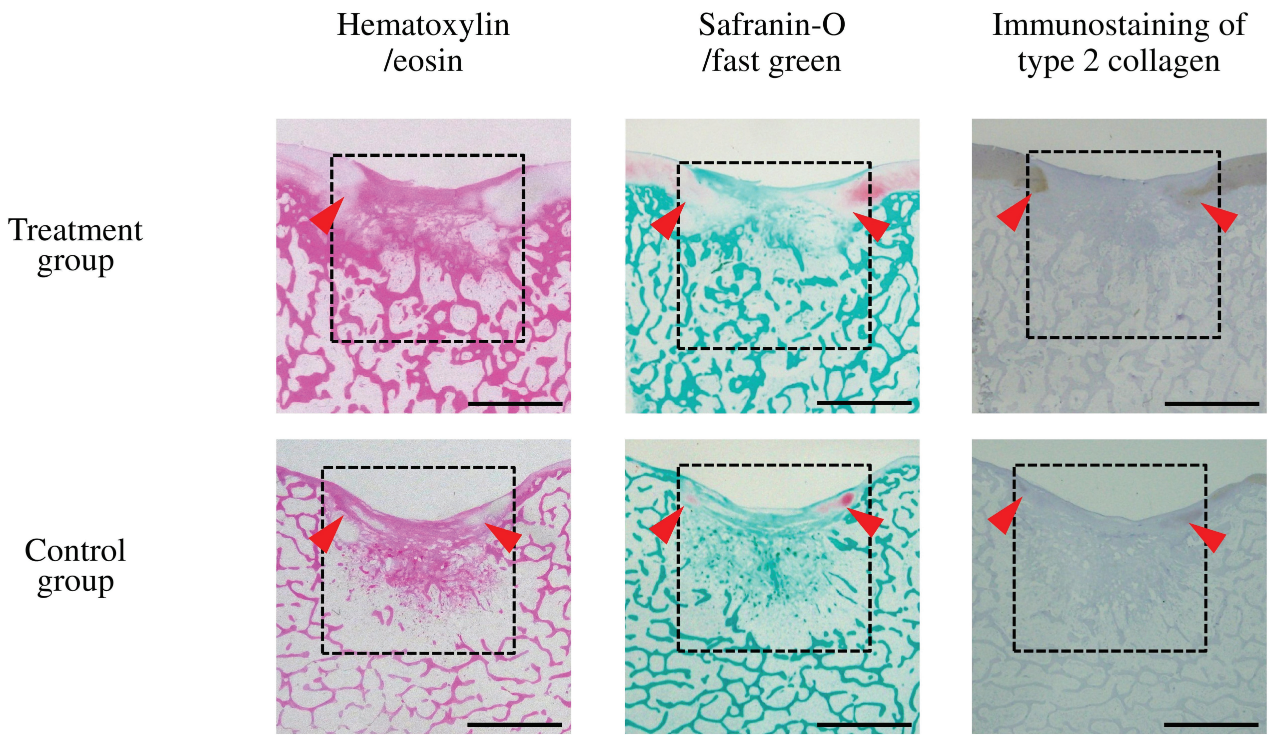

Figure 5. Histological appearance of the cartilage in horse No. 4. The broken line indicates the drill-hole region. The arrowhead indicates that the tissue is positive for safranin-O staining and immunopositive for type 2 collagen. In the treatment group, safranin-O staining and tissue immunopositive for type 2 collagen covered a wider area than observed in the control group. Subchondral bone regeneration was observed over a wider area in the treatment group compared with the control group. The treatment side had a subchondral bone density score of 3, and the control side had a subchondral density score of 0. Upper panel: treatment group, lower panel: control group, Scale bar = 5 mm.

the subchondral bone can result in cartilage damage over time. Thus, the regeneration and restoration of subchondral bone may be necessary for cartilage regeneration. Therefore, the improved subchondral bone regeneration observed in this study may have also resulted in superior cartilage repair.

In contrast to accelerated subchondral bone development and improved macroscopic indices of healing, we found no significant differences in total histological scores or subscores for cartilage healing between the 2 groups. This may have been due to insufficient retention of BMP-2 and PRP-derived factors. An earlier study revealed that 40% of the gelatin ß-TCP sponge remained 30 d after implantation (17). Therefore, we assume that almost all the impregnated sponge had degraded and disappeared from the osteochondral defect site by the end of the 16-week recovery period. Moreover, it has been reported that the drug delivery function of gelatin hydrogel persists for only 2 to 4 wk (5,17,32). Therefore, BMP-2 and PRP possibly disappeared from the osteochondral defect sites during the early stages of regeneration. The lack of a significant difference in cartilage histological score may reflect early disappearance of growth factors from the injury site so that any early improvement in cartilage regeneration was not maintained. Nonetheless, the median total histological score was higher (though not reaching statistical significance) and hyaline cartilaginous tissue was observed across a wider area in the treatment group than in the control group. Thus, cartilage regeneration may have been enhanced modestly in the treatment group, possibly due to an early improvement in cartilage regeneration and enhancement of subchondral bone regeneration. Short-term studies and other case-control studies are required to elucidate the reasons for this relatively modest cartilage repair. Furthermore, significant improvements in these gelatin ß-TCP sponges are still needed, particularly the capacity to sustain any possible early improvement in cartilage regeneration.

In the present study, osteochondral defects were created at the medial femoral condyle, where subchondral bone cysts are commonly found (43). Most cases of subchondral bone cysts are treated by an arthroscopic injection of corticosteroids into the cyst or by arthroscopic debridement of the cyst (43,44). The hole drilled to replicate a condylar defect is similar in size to that needed for arthroscopic debridement. The accelerated regeneration of subchondral bone and modest regeneration of hyaline cartilage suggest that implantation of impregnated gelatin ß-TCP sponges could be a feasible treatment option for subchondral bone cysts.

In conclusion, we fabricated a ß-TCP sponge impregnated with MSC, BMP-2, and PRP and demonstrated that insertion of this sponge into an osteochondral defect promoted osteochondral regeneration. This method could be applied to osteochondral disorders such as subchondral bone cysts. CVJ

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